The Immune Response of Taiwan Abalone *Haliotis diversicolor supertexta* and Its susceptibility to *Vibrio parahaemolyticus* under Environmental Stress

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ABSTRACT

Taiwan abalone held in 30 ‰ seawater and then transferred to 20, 25, 30 and 35 ‰ were examined for immune parameters, and phagocytic activity and clearance efficiency to *Vibrio parahemolyticus* after 24 and 72 h. The THC (total haemocyte count) increased directly with salinity levels. Phenoloxidase activity, respiratory burst, phagocytic activity and clearance efficiency to *V. parahaemolyticus* decreased significantly for the abalone transferred to 20, 25 and 35 ‰. Abalone held in 30 ‰ seawater and 28℃ and then transferred to 20, 24, 28 and 32℃ were examined for immune parameters after 24 and 72 h. The phenoloxidase activity and phagocytic activity decreased significantly for the abalone transferred to 32℃, whereas the THC and respiratory burst increased significantly for the abalone transferred to 32 ℃. The abalone showed decreased immunity and decreased resistance against *V. parahaemolyticus* infection when the animals were subjected to stress both in salinity and temperature.

Key words: Immune response, *Haliotis diversicolor supertexta*, Susceptibility, *Vibrio parahaemolyticus*, Environmental stress.

INTRODCTION

Temperature and salinity are the primary physical factors affecting the life of molluscs. Salinity in the range of 30-35 ‰ and temperature in the range of 24-30℃ are the optimal levels for the growth of Taiwan abalone (Chen, 1984). It is known that Taiwan abalone reared at 30 ‰ and at 20, 25 and 30℃ survive temperature in the range of 4.5-33.1, 7.7-33.7 and 13.5-36.5℃, respectively when water temperature was decreased or increased gradually (Chen and Chen, 1999). Twiwan abalone reared at 30 ‰ and at 20, 25 and 30℃ survive salinity in the range of 18-39, 16-37 and 15-36 ‰, respectively when salinity was decreased or increased gradually (Chen and Chen, 2000).

In molluscs, agranular haemocytes (hyalinocytes) and granular haemocytes (granulocytes) are involved in phagocytosis (Bayne, 1990). Haemocytes are also involved in coagulation and the production of melanin via the prophenoloxidase system (Söderhäll *et al.*, 1996). During phagocytosis, the release of superoxide anion and other ROIs (reactive oxygen intermediates) is known as respiratory burst, and it plays an important role in microbicidal activity.

Taiwan abalone has been mostly cultured in land-based concrete farms adjacent to the sea coast. The salinity in abalone farms may decrease suddenly to as low as 20 ‰ during the rainy season and when typhoons strike. Seasonal range of water temperature in fish farms may vary from 15 to 32℃. Culture of abalone has suffered mass mortality due to vibriosis since 1998 in Taiwan. The bacteria, *Vibrio parahaemolyticus* and *V. alginolytius* isolated from the haemolymph of moribund abalone, have been demonstrated to cause outbreaks of vibriosis associated with warm temperature (Lee *et al.*, 2001).

This study was aimed at determining the immune response of Taiwan abalone under salinity change and temperature change. Total haemocyte count (THC), phenoloxidase activity, respiratory burst (release of superoxide anion), and phagocytic activity of abalone to *V. parahaemolyticus* were examined. Challenge tests were also conducted.

MATERIALS AND METHODS

Taiwan abalone were obtained from a commercial farm were acclimated at 30 ‰ for two weeks at room temperature (26-28 ℃). During the acclimation period, animals were fed red seaweed *Gracilaria tenuistipitata* daily.

V. parahaemolyticus was isolated from diseased abalone. It was cultured on 50 ml tryptic soy broth supplemented with 3 % NaCl (TSB, Difco) for 24 h at 30℃. The stock cultures were centrifuged at 7155 x g for 15 min at 4℃. The supernatant fluid was removed and the bacterial pellet was re-suspended in saline solution at 8 x 10^6 and 8 x 10^8 cfu ml^{-1} as stock bacterial suspension for injection challenge, and the tests of phagocytic activity and clearance efficiency.

Each abalone was injected into the pallial sinus with 20 µl of bacterial suspension (8 x 10^6 cfu ml^{-1}). Challenge tests at a dose of 1.6 x 10^5 cfu $abalone^{-1}$ were conducted in triplicates. The test and control groups comprised 10 abalones each. After injection, abalones were kept in 15 l tanks (10 abalones in each) containing 10 l of aerated water at 20, 25, 30 (control) and 35 ‰. Abalones injected with an equal volume of sterile saline solution and kept in 30 ‰ seawater served as unchallenged controls (Table 1).

For the challenge test on temperature stress, abalones were kept in 15 l tanks (10 abalones in each) containing 10 l of aerated seawater at 20, 24, 28 and 32℃ after injection. Abalones injected with an equal volume of sterile saline solution and kept in 28℃ served as the unchallenged controls (Table 2).

For haemocyte counts and enzyme activity assays, abalones acclimated to 30 ‰ seawater were placed in eight replicates in aquaria with 20, 25, 30 and 35 ‰ seawater that was renewed daily for 5 days. Water temperature was maintained at 26 ± 1℃ (Cheng *et al.*, 2004a). For the temperature stress, abalone acclimated to 30 ‰ seawater at 28℃ were transferred to 20, 24, 28 and 32 ℃. Abalones were sampled individually at the beginning of the test and at 24, 72 and 120 h (Cheng *et al.*, 2004b).

Haemolymph was withdrawn. A drop of diluted haemolymph (50 µl) was placed on a haemocytometer, and THC was measured using an inverted phase contrast microscope. Phenoloxidase activity and respiratory burst were measured.

For phagocytic activity and bacterial clearance tests, abalones after 0, 24, 72 and 120 h exposure at 20, 25, 30 and 35 % and after exposure to 20, 24, 28 and 32 C were injected with 20 µl bacterial suspension (8 x 10^8 cfu ml^{-1}

and 5 x 10^8 cfu ml^{-1}), resulting in 1.6 x 10^7 and 1 x 10^7 cfu $abalone^{-1}$ for salinity stress test and temperature stress test, respectively. After injection, the abalones were held in their respective test solutions for 3 h. Two hundred microliter of haemolymph were collected from the pallial sinus.

Phagocytic activity was measured. Two hundred haemocytes were counted and the phagocytic rate (PR) was expressed as follow:

PR={(Phagocytic haemocytes)/(Total haemocytes)} x 100

Clearance efficiency was measured. Clearance efficiency to *V. parahaemolyticus* was calculated and expressed as percentage inhibition (PI) as follows:

PI = 100- {(cfu in test group)/(cfu in control group)} x 100

A multiple comparison (Tukey) test was conducted to compare the significance of differences among treatments using the SAS computer software (SAS Institute Inc., Cary, NC, USA).

RESULTS

All the unchallenged control abalones survived. By contrast, the onset of mortality occurred at 6 h in the challenged abalone held at a salinity of 20, 25 and 35 ‰. Over 24-120 h, the cumulative mortality for the abalone transferred to 25 ‰ and 35 ‰ was significantly higher than for the abalone held at 30 ‰ seawater (Table 1).

All the abalones transferred to temperature of 32℃ died within 72 h. Over 24-120 h, the cumulative mortality for the abalone transferred to 32℃ was significantly higher than for the abalone held at 28℃ (Table 2).

Table 1. Susceptibility of Taiwan abalone to *Vibrio parahaemolyticus* at different salinity levels at 26±1℃. Data in the same column with different letters are significantly different ($P<0.05$) among treatments. Values are mean±SE (n=30 abalones in each case).

Bacterial dose	Salinity	Cumulative mortality (%)						
(cfu $abalone^{-1}$)	(‰)	6	12	24	48	72	96	120
Control	25	0	0	0	0	0	0	0
1.6×10^5	20	26.7±3.3^a	40.0±5.8^a	70.0±5.8^a	100.0±0.0^a	100.0±0.0^a	100.0±0.0^a	100.0±0.0^a
1.6×10^5	25	10.0±5.8^b	33.3±3.3^{ab}	46.7±3.3^b	56.7±3.3^b	63.3±3.3^b	63.3±3.3^b	63.3±3.3^b
1.6×10^5	30	0.0±0.0^b	3.3±3.3^b	20.0±5.8^c	23.3±3.3^c	36.7±3.3^d	36.7±3.3^d	36.7±3.3^d
1.6×10^5	35	3.3±3.3^b	20.0±5.8^b	40.0±5.8^b	50.0±0.0^b	53.3±3.3^c	53.3±3.3^c	53.3±3.3^c

Table 2. Susceptibility of Taiwan abalone to *Vibroio parahaemolyticus* at different temperatures at 30 ‰. Data in the same column with different letters are significantly different (P< 0.05) among treatments. Values are mean ±S.E (n=30 abalones in each case).

Bacterial dose	Temperature	Cumulative mortality (%) time after challenge (h)					
(cfu $abalone^{-1}$)	(˚C)	6	12	24	48	72	96
Control	28	0	0	0	0	0	0
1.6×10^5	20	0.0±0.0^b	10.0±5.8^c	23.3±3. 3^c	33.3±3.3^c	36.7±6.7^c	36.7±6.7^c
1.6×10^5	24	0.0±0.0^b	16.7±6.7^b	23.3±3.3^c	30.0±5.8^c	33.3±6.7^c	33.3±6.7^c
1.6×10^5	28	3.3±3.3^{ab}	26.7±6.7^b	33.3±6.7^b	53.3±3.3^b	60.0±5.8^b	60.0±5.8^b
1.6×10^5	32	10.0±5.8^a	33.3±3.3^a	56.7±3.3^a	76.7±3.3^a	100.0±0.0^a	100.0±0.0^a

After 24 h, the THC decreased significantly by 49 % and 31 % for the abalone transferred to 20 and 25 ‰ salinity, respectively. After 24 h, phenoloxidase activity decreased significantly for the abalone transferred to 20, 25 and 35 ‰. The respiratory burst decreased significantly for the abalone transferred to 20, 25 and 35 ‰. After 24 h, phagocytic activity decreased significantly to 9 %, 11 % and 13 %, and clearance efficiency decreased by 66 %, 59 % and 72 % for the abalone transferred to 20, 25 and 35 ‰, respectively, compared to the abalones held in 30 ‰.

After 24 h, the THC increased significantly by 22 % and 29 % for the abalone transferred to 20 and 32 ℃, respectively. Phenoloxidase activity decreased significantly for the abalone transferred to 24 and 32 ℃, whereas phenoloxidase activity increased for the abalone transferred to 20℃ after 24, 72 and 120 h. The respiratory burst decreased significantly for the abalone transferred to 20 ℃ after 24 h, whereas the respiratory burst increased significantly for the abalone transferred to 32℃ after 72 and 120 h. After 24 h, phagocytic activity decreased significantly to 10 %, 13 % and 9 % for the abalones transferred to 20, 24 and 32℃, respectively, as compared to the activity of abalones at 28℃.

DISCUSSION

Taiwan abalone was more susceptible to *V. parahaemolyticus* when the animals were transferred to 20 and 25 ‰ from 30 ‰, and when the animals were transferred to 32℃ from 28℃. We may conclude that change in temperature and salinity can trigger disease outbreak by affecting the defense mechanism of the host: the susceptibility of abalone to *V. parahaemolyticus* is enhanced by high temperature (32℃) and low salinity (20 and 25 ‰). All the abalones transferred to 20 ‰ were killed in 48 h. Taiwan abalone maintained in 30 ‰ and at 20, 25 and 30℃ exhibited 96-h LS50 (median lethal salinity) of 22.1, 20.8 and 19.9 ‰ when the abalone were suddenly transferred to a series of low salinity levels (Chen and Chen, 2000). Taiwan abalone which had been maintained in 30 ‰ and at 30℃, and transferred to a series of higher temperature, exhibited 24-h LT50 of 32.2℃ which was higher than the LT50 of present study (31.1℃) (Chen and Chen, 1999)

The total number of circulating haemocytes can be affected by environmental change. Sâo Paulo shrimp *Farfantepenaeus paulensis* reared in 34 ‰ had a significantly higher THC (20 % more) as compared to the shrimp reared at 22 and 13 ‰ (Le Moullac and Haffner, 2000). *M. rosenbergii* reared in 15 ‰ had a significantly higher THC (15 % more) when compared to prawns reared in freshwater (Cheng and Chen, 2000). Similar phenomenon was observed in the present study: the THC of Taiwan abalone increased by 44 % and 56 % for the abalone reared in 30 and 35 ‰, respectively, as compared to the abalone reared in 25 ‰. *M. rosenbergii* reared in 27 and 30℃ had a significantly higher THC as compared to prawns reared in 20 and 33℃. An increase of circulating haemocyte count occurred in the Pacific oyster *Crassostrea gigas* following 15 min mechanic disturbance (Lacoste *et al.*, 2002). The fact that the THC increased for the abalone transferred to 20℃ is considered a consequence of stress response.

In *M. rosenbergii*, phenoloxidase activity, phagocytic activity and clearance efficiency

to *L. garvieae* were significantly higher for animals reared at 5 and 10 ‰ than those reared in freshwater and 15 ‰ (Cheng and Chen, 2000; Cheng et al. 2003). In the present study, the increases of both phenoloxidase activity and respiratory burst are well correlated with the number of THC for abalone in the range of 20-30 ‰. In *M. rosenbergii*, phenoloxidase activity, phagocytic activity and clearance efficiency to *L. garvieae* were significantly higher for animals reared at 27 and 30℃ than those reared in 20 and 33℃. In the present study, phenoloxidase activity increased together with an increase in THC for the abalones transferred to 20℃.

Phagocytosis is affected by environmental parameters in invertebrates (Bayne, 1990). For example, an elevated temperature has been reported to increase phagocytosis (Feng and Feng, 1974) and haemocyte activity (the ability of haemocyte to adhere the fluorescent beads) in the American oyster *C. virginica*. Decrease in salinity has been reported to increase haemocyte activity in *C. vurginica* and in European flat oyster *Ostrea edulis* (Fisher *et al.*, 1987). A reduction in phagocytosis was observed for *Haliotis rufescens* in 35 ‰ and 45 ‰ (Martello *et al.*, 2000). In the present study, phagocytic activity and clearance correlated with increased susceptibility of abalone to *V. parahaemolyticus* when the abalone were transferred to 20, 25 and 35 ‰ from 30 ‰ in 72 h. The phagocytic activity of *V. parahaemolyticus* decreased for the abalone transferred to 20 and 32 ℃ in 24 h.

In conclusion, the susceptibility of abalone to *V. parahaemolyticus* correlated with reductions in immune parameters including phenoloxidase activity, respiratory burst, phagocytic activity and clearance efficiency when the abalone were transferred to 20, 25 and 35 ‰ from 30 ‰ in 72 h. Taiwan abalone transferred from 28 ℃ to 32 ℃ showed higher susceptibility to *V. parahaemolyticus*, and lower phenoloxidase activity and phagocytic activity indicated a reduction in immune ability.

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REFERENCES

Bayne, C.J. 1990. Phagocytosis and non-self recognition in invertebrates. Phagocytosis appears to be an ancient line of defense. Bioscience 40: 723-731.

Chen, H. C. 1984. Recent innovations in cultivation of edible molluscs in Taiwan with special reference to the small abalone *Haliotis diversicolor* and the hard clam *Metretrix lusoria.* Aquaculture 39: 11-27.

Chen J.C. and W.C. Chen. 1999. Temperature tolerance of *Haliotis diversicolor supertexta* at different salinity and temperature levels. Comparative Biochemistry and Physiology 124 A: 73-80.

Chen, J. C. and W. C. Chen. 2000. Salinity tolerance of *Haliotis diversicolor supertexta* at different salinity and temperature levels. Aquaculture 181 : 191-203.

Cheng, W. and J. C. Chen. 2000. Effects of pH, temperature and salinity on immune parameters of the freshwater prawn *Macrobrachium rosenbergii*. Fish & Shellfish Immunology 10: 387-391.

Cheng W., S.M. Chen, F.I. Wang, P.I. Hsu, C.H. Liu and J.C. Chen. 2003. Effects of temperature, pH, salinity and ammonia on the phagocytic activity and clearance efficiency of giant freshwater prawn *Macrobarchium rosenbergii* to *Lactococcus garvieae*. Aquaculture 219: 111-121.

Cheng, W., F.M. Juang and J.C. Chen. 2004a. The immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its suscetibility to *Vibrio parahaemolyticus* at different salinity levels. Fish & Shellfish Immunology 16: 295-306.

Cheng, W., F.M. Juang, C.H. Hsu and J.C. Chen. 2004b. Change in water temperature on the immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its suscetibility to *Vibrio parahaemolyticus*. Fish & Shellfish Immunology 17: 235-243.

Feng, S.Y. and J.S. Feng. 1974. The effect of temperature on cellular reactions of *Crassostrea virginica* to the infection of avian erythrocytes. Journal of Invertebrate Pathology 23: 22-37.

Fisher, W.S., M. Auffret and G. Ballouet. 1987. Response of European flat oyster (*Ostrea edulis*) hemocyte to acute salinity and temperature changes. Aquaculture 67: 179-190.

Lacoste A, S.K. Malham, F. Gélébart, A. Cueff and S. A. Poulet. 2002. Stress-induced immune changes in the oyster *Crassostrea gigas*. Developmental and Comparative Immunology 26: 1-9.

Lee, K.K., P.C. Liu, Y.C. Chen and C.Y. Huang. 2001. The implication of ambient temperature with outbreak of vibriosis in cultured small abalone *Haliotis diversicolor supertexta* Lischke. Journal of Thermal Biology 26: 585-587.

Le Moullac, G. and P. Haffner. 2000. Environmental factors affecting immune response in Crustacea. Aquaculture 191: 121-131.

Martello, L. B., C.S. Friedman and R.S. Tjeerdema. 2000. Combined effects of pentachlorophenol and salinity stress on phagocytic and chemotactic function in two species of abalone. Aquatic Toxicology 49: 213-225.

Söderhäll, K., L. Cerenius and M.W. Johansson. 1996. The prophenoloxidase activating system in invertebrates. In *New Directions in Invertebrate Immunology* (K. Söderhäll, S.G.R. Iwanaga & G.R.Vasta, eds) p. 229-253. Fair Haven, NJ: SOS Publications.